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CROPS AND SOILS RESEARCH PAPER

Barley seed coating with free and immobilized alkaline phosphatase to improve P uptake and plant growth

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SUMMARY

Coating barley seeds with free and immobilized alkaline phosphatase was investigated as a potential means to enhance plant utilization of accumulated soil phosphorus (P). Two coating techniques were studied: film-coating and pelleting. The highest phosphatase activity retention in the coating layer, ranging from 0·48 to 0·67, was observed when seeds were film-coated with phosphatase–polyresorcinol complex (PPC). The germination of seeds film-coated or pelleted with alkaline phosphatase ranged from 0·84 to 0·97 or 0·14 to 0·25, respectively. Low germination of the pelleted seeds was attributed to freezing the seeds in liquid nitrogen (N) for the layer coating formation. Pelleted seeds were not used in the remainder of the studies. Under pot culture conditions, an increase in the soil inorganic P was detected when the seeds were film-coated with phosphatase. Moreover, the film-coating significantly increased the P uptake by plants (between 25 and 31% after 35 days after planting (DAP)). The present study showed that the seed film-coating with free and immobilized phosphatase increased the phosphatase activity in the rhizosphere and the P uptake by plants.

INTRODUCTION

Deficiency of soil phosphorus (P) is a universal constraint to crop production and constitutes the second most important soil fertility problem throughout the world. A large quantity of soluble forms of P fertilizers is applied to achieve maximum plant productivity. The P fertilizer utilization efficiency should be considered when making P fertilizer recommendations (Tang *et al*. 2011). However, a great part of the soluble P introduced into soil as fertilizer reacts with soil components, forming insoluble P products (Vassileva *et al*. 2000), which are not efficiently taken up by the plants (Omar 1998). This unmanaged excess of P application is known to cause economic and environmental problems. Thus, a high P concentration in soil can lead to eutrophication of natural water reservoirs due to soil erosion and runoff water (Carpenter 2005).

Organic P represents 0·20–0·80 of the total P in most soils and consequently represents a significant reserve of potentially available P (Fransson & Jones 2007). Although some forms of organic P may be taken up directly by plants and micro-organisms, there is a general agreement that most organic phosphocompounds must first be mineralized through hydrolysis catalysed by extracellular phosphatases. The activity of these enzymes plays a key role in soil P cycling and plant nutrition (Tarafdar *et al*. 2003).

Increased P bioavailability was studied by inoculation of P-solubilizing bacteria (Canbolat *et al*. 2006; Mishra *et al*. 2008) and P seed coating (Peltonen-Sainio *et al*. 2006; Sekiya & Yano 2010). Burns *et al*. (1988) suggested the use of a stable phosphatase seed coating as a tool to enhance P bioavailability. Busto & Perez-Mateos (1997) reviewed the relationship between soil enzymes, environment and fertility, including some biotechnological application of immobilized enzymes.

A definition of seed coating is 'a general term for the application of finely ground solids or liquids containing dissolved or suspended solids to form a more or less continuous layer covering the natural seed coat' (Scott 1989). Seed coating can be employed to change the physical form, i.e. to facilitate the mechanical sowing, to increase seed weight for air-seeding purposes and to apply chemical seed treatment formulations, including chemical pesticides, biological

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pesticides or germination enhancers, nutrients or plant growth regulators (Scott 1989; Taylor *et al.* 1998; Halmer 2000; Ziani *et al.* 2010). The two main methods for seed coating are pelleting and film-coating. Pelleting is defined as the deposition of a layer of inert materials that can change the original shape and size of the seed, resulting in a substantial weight increase. Film-coating, however, retains the shape and the general size of the raw seed with minimal weight gain (Taylor *et al.* 1998).

Pilar *et al.* (2009) observed that the encapsulation of barley seeds in calcium alginate hollow beads with a stable phosphatase–polyresorcinol complex (PPC) exhibited high rhizosphere phosphatase activity, resulting in a positive effect on phosphate uptake and plant growth. In the present paper, the coating of barley seed by film-coating and pelleting with both free and immobilized alkaline phosphatase is presented as a potential means to enhance plant utilization of accumulated soil P. The effects of barley seed coating on soil phosphatase activity, seed germination, plant growth and P uptake were also investigated. Specifically, the present study may help in development of sustainable agriculture, avoiding the deleterious effects of excessive P fertilizer application and maintaining adequate crop yield.

MATERIALS AND METHODS

Experimental design

Barley seeds, *Hordeum vulgare* L. cvar Volley, were coated with free and immobilized alkaline phosphatase (EC 3.1.3.1). The enzymatic solution was obtained, as described Pilar *et al.* (2003), from *Escherichia coli* ATCC27257, a phosphatase hyperproductive strain obtained from the Spanish Official Culture Collection (Valencia, Spain). Phosphatase was immobilized by co-flocculation with humate and by phenolic co-polymerization with resorcinol. The coating methods applied were film-coating, using methylcellulose (MC) as a binder and pelleting with poly (vinyl alcohol) (PVA) as a filler. First, the binder or filler concentration and the enzyme/binder or enzyme/filler relation were optimized for each seed coating. The optimal conditions were selected in order to achieve adequate levels of phosphatase activity in the coating layer, avoiding seed agglomeration and fragmentation of the coating. A germination test was performed to assess the viability of seeds treated. Seeds with low germination were discarded for the pot tests.

A pot culture experiment was carried out to study the effects of the coating on the plant growth and P uptake. The soil used in the experiments was collected from the surface (0–100 mm) from a farm field at Ribera del Arlanza in Burgos (Spain). Prior to use, the soil was air-dried at room temperature (22 ± 1 °C) and then gently ground to pass through a 2 mm sieve. Relevant soil properties are: pH 8·7, organic C 5·93 g/kg, C:N ratio 7·15, organic P 0·27 g/kg and inorganic P 0·52 g/kg. Soil pH was measured in 1:5 (w/w) soil: H_2O extract. Soil organic carbon was determined following the wet digestion method of Walkley & Black (1934). C:N ratio was determined using an elemental analyser (CHN A-932, LECO, St. Joseph, MI, USA). Inorganic P and organic P were estimated following the method reported by Saunders & Williams (1955). Seeds were germinated on a wet filter paper at 26 °C in a growth chamber (Conviron® E7) for 96 h. Five seedlings were transplanted to a plastic pot (containing 150 g of soil) at 10 mm below the soil surface, and then the pots were transferred to an artificial climatic chamber. The plants were grown under environmental conditions of 20/15 °C light/dark period temperature and a 16 h photoperiod per day. The pots were watered everyday with 15 ml of water. Each experiment was conducted five times. One plant from each replicate was carefully freed from soil at 7, 14, 21, 28 and 35 days after planting (DAP). After each harvest, the plant growth was determined by measuring the length, dry weight and P concentration of the shoot. Parallel soil samples were taken in order to evaluate the inorganic P and organic P, as described by Saunders & Williams (1955), and phosphatase activity in the bulk soil.

Alkaline phosphatase immobilization

Immobilization in soil humates

The immobilization of alkaline phosphatase by co-flocculation with soil humates in the presence of Ca^{2+} was as described Pilar *et al.* (2003). The immobilized enzyme was frozen and lyophilized. The insoluble humate–phosphatase complex (HPC) resuspended in 2-amine-2-methyl-1propanol (2A2M1P) (15 mg/ml) was assayed separately for alkaline phosphatase activity.

Preparation of the alkaline PPC

Alkaline PPC was synthesized following the method reported by Pilar *et al.* (2009). The copolymer was frozen and lyophilized. The insoluble PPC

Table 1. *Enzyme/binder and enzyme/filler ratios assayed in the seed film-coating and seed pelleting process, respectively*

Enzyme/binder ratio*			Enzyme/filler ratio†		
Free phosphatase (ml/ml)	HPC (g/ml)	PPC (g/ml)	Free phosphatase (ml/ml)	HPC (g/ml)	PPC (g/ml)
0·04	0·07	0·01	0·04	0·07	0·01
0·10	0·13	0·02	0·08	0·13	0·02
0·20	0·20	0·04	0·12	0·20	0·04
0·50	0·27	0·06	0·16	0·27	0·06
1·00	–	–	–	–	–

* Seed film-coating: each experiment was conducted at 0·02, 0·03, 0·04, 0·05 and 0·06 g/ml MC.
† Seed pelleting: each experiment was conducted at 0·04, 0·06 and 0·08 g/ml PVA.

resuspended in 2A2M1P (2·5 mg/ml) was assayed separately for alkaline phosphatase activity.

Alkaline phosphatase assay

The activity of alkaline phosphatase was assayed by using *p*-nitrophenyl phosphate (*p*NPP) (Sigma, St. Louis, MO, USA) as an artificial substrate (Tabatabai & Bremner 1969). Activity of both immobilized and free enzyme was determined by incubating 1 ml of 25 mmol/l *p*NPP, 3 ml of 0·1 mol/l 2A2M1P (pH 10·5, containing 1 mmol/l $MgSO_4 \cdot 7H_2O$ and 2 mmol/l $ZnSO_4 \cdot 7H_2O$), and 1 ml of the enzyme solution at 37 °C for 60 min. For the phosphatase activity of coated seeds and soil, samples of six seeds (mean weight of 0·29 ± 0·01 g) and 1 g of fresh soil, respectively, were added to 4 ml of 0·1 mol/l 2A2M1P buffer at pH 10·5. Thereafter, 5 ml of 0·5 mol/l NaOH and 1 ml of 0·5 mol/l $CaCl_2$ were added to the mixtures (to flocculate the organic matter and extract the *p*-nitrophenol (*p*NP)), first shaking and then allowing standing for 10–15 min at 20 °C. The contents were filtered (Whatman No. 6) and the *p*-nitrophenol released was measured at 410 nm using a spectrophotometer (Hitachi U-2000). In parallel, a calibration curve using concentrations of *p*NP in the range 0–20 µg/ml was run. Controls, the substrate of which was added after incubation, were assayed in all cases to deduct any non-enzymatic activity. Phosphatase activity was referred to 1 ml of enzyme solution, 1 g of immobilized enzyme, 1 g of coated seed or 1 g of dry weight soil.

Techniques for seed coating with alkaline phosphatase

Prior to coating, the barley seeds were surface-sterilized by washing with 50 mg/ml sodium hypochlorite for 1 min, followed by rinsing five times with sterile distilled water. Then, the seeds were placed on filter paper and dried by a current of sterile air in a laminar flow cabinet Bio-II-B (Telstar®).

Film-coating

Barley seeds were film-coated with a solution containing MC (Sigma, St. Louis, MO, USA) and free or immobilized enzymes. MC concentrations and enzyme/binder ratio tested for free and immobilized enzyme are detailed in Table 1. The seeds were coated with each coating solution by the following procedure: 3 g of seeds were added to 1 ml of the coating solution with free phosphatase or PPC or to 1·5 ml of the coating solution containing HPC. The mixture was agitated to accomplish uniform coating of the seed surface. Then, seeds were carefully placed, one by one, on filter paper in Petri dishes and dried under sterile airflow in a laminar flow cabinet.

Pelleting

Barley seeds were pelleted using PVA as coating agent. The PVA used had a number average molecular weight, M_n, of 72 000, a degree of polymerization of 1600 and a saponification value >97·5 (Sigma, St. Louis, MO, USA). PVA and sodium hydroxide (NaOH, 1:0·025 w/w) were dissolved in 16 ml deionized water by autoclaving for 15 min at 121 °C, cooled to room temperature, neutralized with hydrochloric acid (HCl, 350 g/kg), mixed with free or immobilized phosphatase and diluted to 25 ml with water. Coating solutions with different PVA concentrations and enzyme/filler ratios were prepared (Table 1). Three grams of seeds were mixed with 4·5 ml of each pelleting solution, and added (one by one) into liquid nitrogen (N), whereupon seeds were

coated with PVA instantly. After 1 h, the liquid N was decanted and the seeds slowly warmed to 5 °C. The pelleted seeds were washed with water for 1 min, and placed on filter paper under sterile airflow in a laminar flow cabinet to dry.

Coating phosphatase activity

The coating phosphatase activity was defined by the ratio of the phosphatase activity of the coated seeds to the activity of the free or immobilized enzyme used in the coating process.

Germination test

The germination test assayed was an unofficial test based on the physiological criterion of radicle emergence (Taylor *et al*. 2001). Germination trials were performed by placing seeds (coated and non-coated) in Petri dishes on filter paper moistened with distilled water and incubated in a growth camber (Conviron® E7) at 26 °C for 96 h. Five replicates of 50 seeds per treatment were used. Seeds were considered as germinated when the radicle was visible.

Rhizosphere phosphatase activity

In order to determine the effect of the seed coating on the rhizosphere phosphatase activity, a qualitative visualization method was used (Trolldenier 1992). The procedure was described in detail in Pilar *et al*. (2009). Briefly, barley seeds pre-germinated for 3 days were placed in agar plates containing phenolphthalein phosphate and kept at room temperature for 15 h. Then, the medium was adjusted to a higher pH so that bright purple-coloured zones occurred on the roots, indicating enzymatic hydrolysis of the reagent.

Plant growth

The length, dry weight and P concentration of the shoot in plants grown from seeds film-coated with free and immobilized phosphates were determined. Dry weight was determined by oven-drying the shoots at 70 °C for 48 h. Shoot samples were subsequently ashed (16 h at 550 °C) and the residue dissolved in 0·9 mol/ml H_2SO_4 (*c*. 1 ml for each 10 mg of oven-dry tissue weight) (Hayes *et al*. 2000). The inorganic P content of the acid solutions was then measured according to the ascorbic acid-molybdate method of Murphy & Riley (1962). Plant P uptake was calculated by multiplying dry weight by P concentration of the shoot.

Statistical procedure

Analysis of variance and regression analysis were performed by Statgraphics Centurion XVI.I for Windows. All determinations were replicated five times. The standard errors of the mean (S.E.M.) are given in Tables and Figures. The standard errors of difference (S.E.D.) were calculated to test differences in phosphatase activity of soil, inorganic and organic P in soil and plant growth between seeds film-coated and control (soil planted with untreated seeds).

RESULTS

Seed coating

The coating phosphatase activity of barley seeds film-coated with free and immobilized phosphatase as a function of the MC concentration and enzyme/binder ratio is shown in Fig. 1. The highest values, ranging from 0·48 to 0·67 (average 0·55), were obtained for seeds coated with PPC. The coating phosphatase activity decreased and ranged from 0·33 to 0·45 and 0·17 to 0·28 with an average of 0·41 and 0·24 for seeds coated with HPC and free enzyme, respectively. Fragmentation of the coating layer was observed in seeds treated with HPC at enzyme/binder ratio of 0·20 and 0·27 g/ml. Moreover, the film-coating at 0·05 and 0·06 g/ml MC promoted seed agglomeration during coating.

Barley seed pelleting with free and immobilized phosphatase, using PVA as a filler, were also investigated (Fig. 2). The highest coating phosphatase activity was achieved with PPC (ranging from 0·39 to 0·44). The average yields for seed treated with free and immobilized enzyme by humates (HPC) and copolymerization (PPC) were 0·18, 0·26 and 0·41, respectively. The coating phosphatase activity was not significantly affected by PVA concentration, except for 0·04 g/ml PVA in seed pelleted with free phosphatase, resulting from the low viscosity of the polymer solution (enzyme—PVA mixture). The seeds easiest to handle were those coated with 0·08 g/ml PVA.

Effect of coating in seed germination

Coating with free phosphatase and PPC did not affect germination, in comparison with untreated seeds

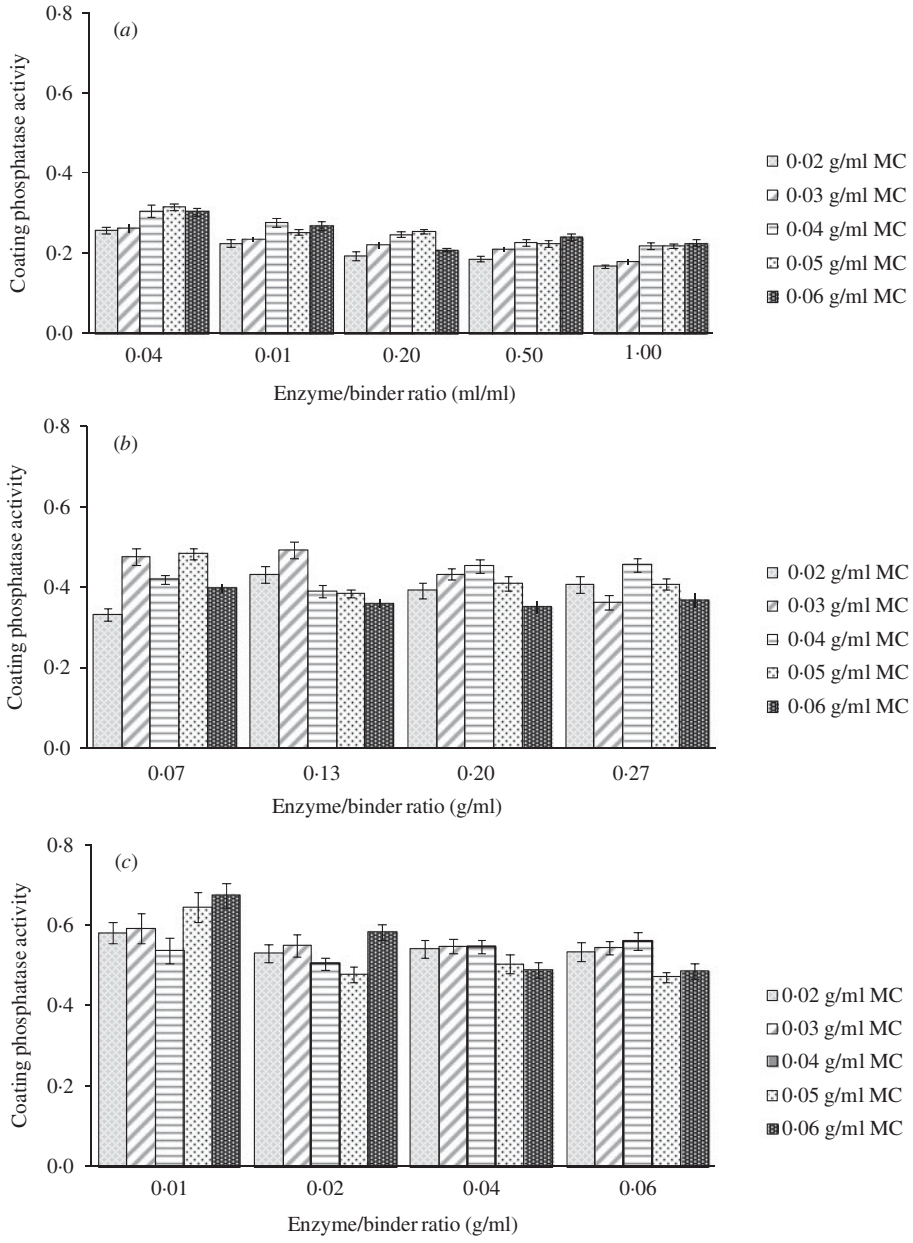

Fig. 1. The effect of enzyme/binder ratio at 0·02, 0·03, 0·04 and 0·06 g/ml MC on the coating phosphatase activity of seeds treated with free phosphatase (14 738 µg *p*NP/ml/h) (*a*), HPC (3728 µg *p*NP/g/h) (*b*) or PPC (14 268 µg *p*NP/g/h) (*c*). Bars represent S.E.M. ($n = 5$).

(Table 2), whereas a slight decrease (about 16%) was observed in seeds coated with HPC. Germination for seeds pelleted with PVA was between 0·14 and 0·25.

Effect of seed coating on rhizosphere phosphatase activity and soil phosphorus mobilization

A higher rhizosphere phosphatase activity was observed in seedlings grown from coated seeds, showing a more intense pink colouration around the roots than the control. As an example of the results obtained, Fig. 3 shows that the colouration around the roots of seeds film-coated with PPC was more intense than that of roots of untreated seeds. No differences were detected in seed coating with free or immobilized enzyme.

The phosphatase activity of the control and soil with film-coated seeds is shown in Table 3. At 35 DAP, no significant differences were detected between the control and soils planted with seeds coated with free phosphatase. In contrast, the phosphatase activity

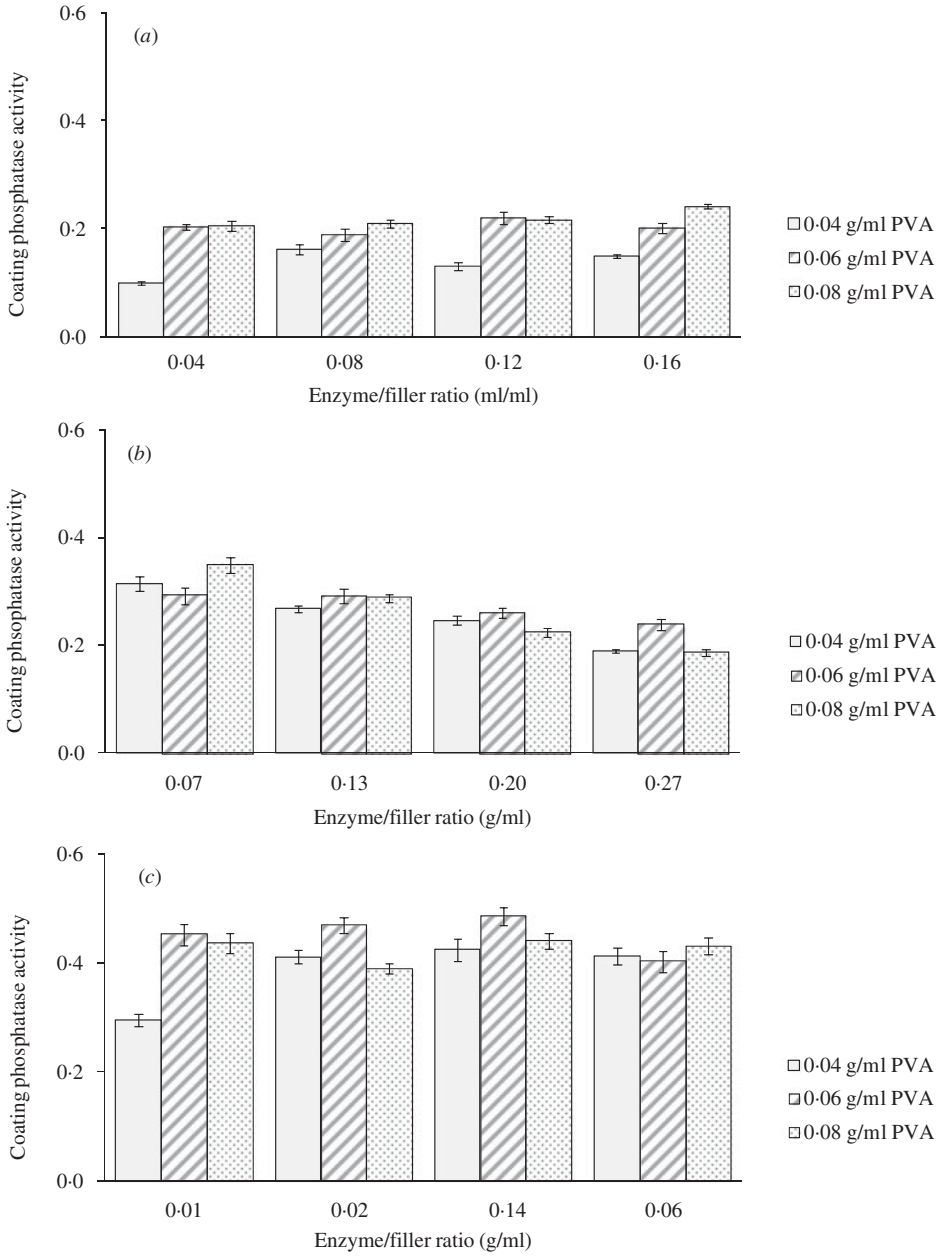

Fig. 2. The effect of enzyme/filler ratio at 0·04, 0·06 and 0·08 g/ml PVA on the coating phosphatase activity of seeds treated with free phosphatase (13 369 μg *p*NP/ml/h) (*a*), HPC (3562 μg *p*NP/g/h) (*b*) or PPC (15 896 μg *p*NP/g/h) (*c*). Bars represent S.E.M. ($n = 5$).

compared to the control was 12·5 and 11·5% lower in soil planted with seeds coated with HPC and PPC, respectively.

The effects of soil planted with film-coated seeds on organic and inorganic P were assessed (Table 4). A decrease of the inorganic P (16·7, 7·2, 6·8 and 9·9%) and an increase of the organic P (54·2, 30·6, 30·9 and 27·3%) were observed in both the control and soils planted with seeds coated with free, HPC and PPC, respectively. After 35 DAP, the pots planted with coated seeds showed higher inorganic P (8–11%) and lower organic P (15–17%) concentrations than the control (non-coated seeds). No significant differences among seeds coated with free or immobilized enzyme were detected.

Effect of seed coating on plant growth and P uptake

The effect of seed coating with free and immobilized phosphatase on P uptake and plant growth is shown in

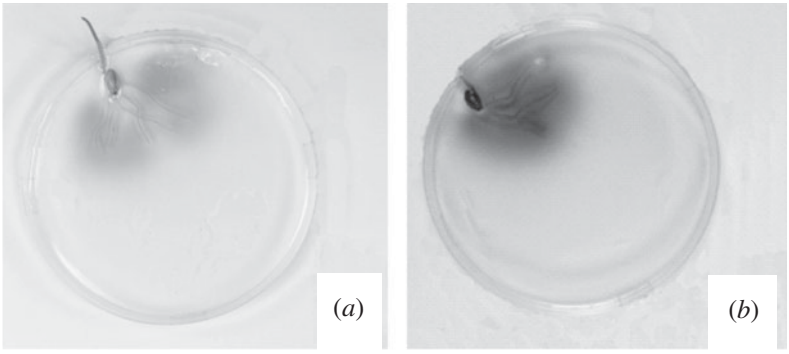

Fig. 3. Barley seedling growing in agar with phenolphtalein phosphate. Seed untreated (control) (*a*) or seed film-coating with PPC (*b*).

Table 2. *Germination test of seeds coated with free or immobilized (HPC and PPC) phosphatase. Values are presented as the mean (n = 5) and* S.E.M. *(in parenthesis)*

	Germination (proportion)*	
Enzyme	Film-coating	Pelleting
Free	0·95 (0·017)	0·25 (0·049)
HPC	0·84 (0·016)	0·15 (0·027)
PPC	0·98 (0·009)	0·14 (0·029)

* In relation to untreated seeds. Control germination: 0·87 (0·009).

Table 3. *Phosphatase activity of soil planted with untreated seeds (control) and seeds film-coated with free or immobilized (HPC and PPC) phosphatase. Values are presented as the mean (n = 5) and* S.E.M. *(in parenthesis)**

	Phosphatase activity (μg *p*NP/g dried soil/h)			
Time (DAP)	Control	Free phosphatase	HPC	PPC
7	85 (3·6)	83 (3·3)	75 (2·8)	73 (1·3)
14	81 (4·3)	78 (3·2)	71 (1·1)	72 (2·3)
21	64 (3·0)	68 (2·2)	65 (3·1)	64 (0·8)
28	71 (3·6)	76 (4·5)	66 (3·0)	63 (2·5)
35	73 (2·4)	68 (2·7)	64 (3·5)	64 (3·0)
S.E.D. (8 D.F.)†		ns	4·19	3·90

* Soil phosphatase activity at $t = 0$: 72·09 (1·60) μg *p*NP/g dried soil/h.
† S.E.D. to compare the coated seeds and the control at 35 DAP.
ns, not significant; D.F., degrees of freedom.

Table 5. Seed coating did not affect the visual aspect of the plant, showing the highest growth during the 21 first days. Plants from seeds coated with phosphatase showed similar shoot length compared to the control. The seed coating with free phosphatase significantly ($P < 0·05$) increased shoot dry weight, by 14%, at 35 DAP. The shoot P content was significantly higher in plants from seeds film-coated with free phosphatase ($P < 0·05$) and with PPC ($P < 0·01$) than in control plants (Table 5), showing an increase of 26 and 28%, respectively, after 28 DAP. In addition, the coating with PPC improved the plant P concentration, resulting in increases of *c*. 20% after 21 and 35 DAP. Phosphorus content in shoots was significantly correlated with phosphatase activity in soil (non-treated seeds, $r = 0·713$, $P < 0·005$; seeds coated with free phosphatase, $r = 0·749$, $P < 0·005$; seeds coated with HPC, $r = 0·711$, $P < 0·005$; seeds coated with PPC, $r = 0·735$, $P < 0·01$). In addition, a decrease in shoot P concentration with time was observed in all pots. Finally, the barley seed coating with free and immobilized phosphatase significantly increased ($P < 0·01$) the P uptake by plants. Thus, after 35 DAP, the plants grown from coated seeds showed an increase in the P uptake ranging from 25 to 31% in comparison with non-treated seeds (Table 5).

DISCUSSION

Coating of seeds with fertilizer or other active ingredients prior to sowing has large potential benefits (Liu & Lister 1993). The nutrient is available precisely where it is required and, in addition, seed coating can also reduce the rate of fertilizer application required. Phosphorus seed coatings have been studied to

Table 4. *Influence of the seed coating with free and immobilized (HPC and PPC) phosphatase on the dynamic of inorganic P and organic P fraction in soil planted with untreated seeds (control) and seeds film-coated with free phosphatase, HPC or PPC. Values are presented as the mean* (n = *5) and* S.E.M. *(in parenthesis)**

				Seed coating	
Soil P	DAP	Untreated	Free phosphatase	HPC	PPC
Inorganic P (mg P/g dried soil)	7	0·50 (0·046)	0·62 (0·024)	0·55 (0·015)	0·58 (0·028)
	14	0·45 (0·024)	0·47 (0·012)	0·53 (0·030)	0·51 (0·010)
	21	0·46 (0·010)	0·50 (0·024)	0·52 (0·011)	0·54 (0·008)
	28	0·45 (0·013)	0·50 (0·021)	0·53 (0·008)	0·56 (0·028)
	35	0·44 (0·004)	0·49 (0·012)	0·49 (0·023)	0·47 (0·010)
S.E.D.(8 D.F.)*†			0·013	0·023	0·010
Organic P (mg P/g dried soil)	7	0·28 (0·011)	0·17 (0·008)	0·24 (0·006)	0·19 (0·004)
	14	0·36 (0·012)	0·44 (0·030)	0·30 (0·010)	0·32 (0·004)
	21	0·38 (0·011)	0·33 (0·010)	0·32 (0·007)	0·31 (0·008)
	28	0·33 (0·006)	0·37 (0·011)	0·29 (0·005)	0·29 (0·012)
	35	0·42 (0·010)	0·35 (0·009)	0·36 (0·017)	0·35 (0·009)
S.E.D.(8 D.F.)*†			0·014	0·020	0·014

* Inorganic P at $t = 0$: 0·526 (0·021) mg P/g dried soil. Organic P at $t = 0$: 0·271 (0·006) mg P/g dried soil.
† S.E.D. to compare the coated seeds and the control at 35 DAP. D.F. = degrees of freedom.

determine whether this increases P bioavailability for plants. Ros *et al.* (2000) analysed the effect of coating and of soaking rice seed with various P fertilizers on early plant growth. Peltonen *et al.* (2006) found that coating the seeds with soluble P fertilizer mixed with a biodegradable fixing agent improved P use, resulting in enhanced early root and seedling growth. An alternative approach, presented in the present paper, is to coat seeds with stable phosphatase as a means of bringing about a localized solubilization of organic phosphate, particularly important in the early stages of seedling establishment.

Barley seeds were film-coated and pelleted with free and immobilized phosphatase. The MC concentration affects of the quality of the coating layer. Scott *et al.* (1997), studying the use of the adhesive Methocel K35 (a low-viscosity MC) on lucerne seeds, observed an increase in the percentage agglomeration at higher MC concentration. A higher viscosity is frequently observed with higher polymer concentrations, resulting in seed agglomeration during the particle coating process (Chen *et al.* 2009). Moreover, the non-spherical shape of barley seeds increases agglomeration (Liu & Lister 1993). Therefore, an MC concentration of 0·04 g/ml was set up to avoid seed agglomeration. The enzyme/binder ratios selected were 1 ml/ml (free phosphatase), 0·20 g/ml (HPC) and 0·06 g/ml (PPC). When seeds were pelleted with PVA, the highest coating phosphatase activity was also achieved with PPC, although this was lower than that obtained with the film-coating. For this technique the optimal conditions were an enzyme/filler ratio of 0·16 ml/ml (free enzyme), 0·27 g/ml (HPC) and 0·06 g/ml (PPC) at 0·08 g/ml PVA.

In many coated seeds, germination and subsequent seedling growth can be inhibited by the mechanical restriction exerted by the seed coating (Sung & Chiu 1995). Moreover, the compounds present in the coating layer applied to the seed surface can also affect germination. Thus, the decrease in germination rate observed in seed film-coated with HPC could be related with the chemical nature of the enzymatic complexes. In this way, Muscolo *et al.* (2001) reported that phenolic compounds extracted from soil inhibited the germination of *Pinus laricio* seeds, and the effects were variable with regard to the phenolic compounds utilized and their composition. The adverse effect of the coating on germination of pelleted seeds was attributed to the freezing of seeds in liquid nitrogen for gel formation. Seed cryopreservation studies revealed that seed viability is directly related to water content, because the ice crystals formed during freezing could produce cellular damage and therefore a decrease in germination (Wood *et al.* 2003). Taking into account the loss of seed viability of pelleting using PVA, film-coating using MC was selected to study the effect of

Table 5. *Influence of the seed coating with free or immobilized phosphatase (HPC and PPC) on the plant growth (dry weight, length and P content of shoots) and P uptake. Values are presented as the mean* (n = *5) and* S.E.M. *(in parenthesis)*

Seed coating	DAP	Shoot dry weight (mg/plant)	Shoot length (mm)	Shoot [P] (µg/mg dry weight)	P uptake (µg/plant)
Non-treated	7	14 (0·4)	154 (6·0)	7·3 (0·21)	100 (4·6)
	14	17 (0·3)	191 (9·2)	7·1 (0·26)	123 (5·6)
	21	30 (1·2)	246 (6·4)	4·8 (0·14)	144 (2·9)
	28	44 (2·2)	265 (6·2)	3·4 (0·12)	151 (10·7)
	35	46 (1·5)	262 (6·7)	2·9 (0·12)	135 (1·6)
Free phosphatase	7	12 (0·4)	139 (6·4)	8·2 (0·21)	99 (2·6)
	14	19 (0·9)	172 (5·4)	6·6 (0·41)	123 (10·0)
	21	30 (2·9)	229 (13·7)	5·2 (0·12)	156 (12·1)
	28	48 (0·7)	256 (6·4)	4·3 (0·12)	209 (3·6)
	35	53 (2·2)	270 (6·5)	3·4 (0·12)	178 (4·0)
HPC	7	13 (0·6)	137 (5·3)	7·1 (0·18)	92 (1·6)
	14	22 (0·7)	180 (4·4)	6·5 (0·20)	142 (4·4)
	21	30 (0·4)	232 (11·9)	5·0 (0·20)	150 (5·4)
	28	48 (2·6)	256 (6·9)	4·4 (0·20)	210 (12.0)
	35	49 (1·5)	250 (7·7)	3·4 (0·08)	168 (7·3)
PPC	7	14 (0·6)	408 (5·6)	9·4 (0·26)	127 (8·8)
	14	24 (1·0)	146 (5·1)	5·9 (0·14)	140 (5·3)
	21	34 (1·7)	246 (9·8)	4·5 (0·22)	152 (4·3)
	28	43 (0·6)	258 (2·6)	4·1 (0·11)	179 (6·7)
	35	50 (3·8)	243 (8·3)	3·5 (0·09)	176 (8·9)
S.E.D. (8 D.F.)*		2·7	ns	0·17	4·3
S.E.D. (8 D.F.)†		ns	ns	0·14	7·4
S.E.D. (8 D.F.)‡		ns	ns	0·15	9·1

* S.E.D. to compare the coated seeds with free phosphatase and control 35 DAP.
† S.E.D. to compare the coated seeds with HPC and control 35 DAP.
‡ S.E.D. to compare the coated seeds with PPC and control 35 DAP.
ns, not significant.

coating on rhizosphere phosphatase, plant growth and P uptake.

Film-coated seeds showed higher rhizosphere phosphatase activity in comparison with the control. The enzyme activity observed in the controls was associated with acid phosphatase activity released by roots. Likewise, as the barley seedlings were ground under non-sterile conditions, some of the phosphatase activity observed could be released for micro-organisms attached to the root surface (Trolldenier 1992). However, planting film-coated seeds did not increase the enzymatic activity in the bulk soil (non-rhizosphere) (Table 3). Therefore, seed film-coating was mainly effective in the root vicinity. Nevertheless, this qualitative method of evaluating the rhizosphere phosphatase did not allow differences in seeds treated with free or immobilized enzyme to be detected.

In the present study, a slight decrease in the inorganic P and an increase in organic P were observed with time in all pots. It is generally accepted that organic P plays an important role in P nutrition of plants; however, there was a discrepancy concerning the change of organic P in the rhizosphere of plants (Li *et al*. 2008). Organic P accumulation in the rhizosphere could be caused by the immobilization of inorganic P forms into organic P forms by micro-organisms in the rhizosphere, whose activity was higher in this zone, mainly because of their utilization of organic anions secreted by plants (Ryan *et al*. 2001; George *et al*. 2002). Alternatively, organic P depletion in the rhizosphere could be explained by the fact that organic P hydrolysis by phosphatase was higher than the immobilization of inorganic P into organic P forms (George *et al*. 2002). In fact, phosphatase in the rhizosphere was observed to be closely related to

depletion or utilization of organic P in the rhizosphere of the plant (Asmar *et al*. 1995; Nuruzzaman *et al*. 2006). Thus, the pots planted with film-coated seeds (with high rhizosphere activity) showed higher inorganic P and lower organic P than soil planted with non-coated seeds. This higher inorganic P accumulation could explain the lower phosphatase activity observed in soil planted with film-coated seeds, since phosphate competitive inhibit the alkaline phosphatase (Pilar *et al*. 2009).

The results obtained in the present pot culture experiment showed the beneficial effect of the seed film-coating with phosphatase by increasing the biomass, the P content of shoot and the P uptake. These results can be related to higher rhizosphere phosphatase activity of film-coated seeds. Solaiman *et al*. (2007) reported that in canola cultures, shoot P content was significantly positively correlated with phosphatase activity in rhizosphere, root length and pH. The decrease observed in the P content of shoot has been reported in barley and other annual species (Hoppo *et al*. 1999; Bolland & Brennan 2005; Ziadi *et al*. 2008), as a result of the higher internal P requirements of annual plants in the early growth. Hoppo *et al*. (1999) suggested that the decrease in shoot P content could be attributed to a dilution effect caused by the increase in the shoot dry weight.

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